

# **Oral administration of type II collagen suppresses pro-inflammatory mediators production by synoviocytes in rats with adjuvant arthritis**

**Key words:** type II collagen; oral tolerance; adjuvant arthritis; synoviocyte; TGF -  $\beta$

**Running title:** Oral collagen II suppresses synoviocyte function

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**Sources of support:** Education Committee Foundation of Anhui Province, Research Foundation of Qilu Pharmaceutical Company in China

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## SUMMARY

**Objective:** To investigate the effect of oral administration of type II collagen (CII) on pro-inflammatory mediator production by synoviocytes in rats with adjuvant arthritis (AA).

**Methods:** Sprague-Dawley rats were fed with bovine CII either before immunization with Complete Freund's adjuvant (CFA) or after initiation of arthritis. Hind paw secondary swelling was measured and synoviocytes were harvested. Sera from portal vein of oral tolerized rats were collected and *in vitro* synoviocytes culture or synoviocytes-Peyer's Patches (PP) cells co-culture system were developed.

Interleukin (IL)-1 activity was measured by a mouse thymocyte activation assayed by MTT dye reduction and tumour necrosis factor (TNF) activity was measured by an L929 cytotoxicity bioassay. Nitric oxide (NO) and malondialdehyde (MDA) levels were measured by biochemical methods.

**Results:** CII 5, 50 and 500µg/Kg feeding for 7 days before immunization significantly suppressed hind paw secondary swelling measured at day 16, 20, 24 and 28 (all  $P<0.001$ ) and pro-inflammatory mediator (IL-1, TNF, NO and MDA) production by synoviocytes (all  $P<0.01$ ) in rats with AA. CII 5, 50 and 500µg/Kg feeding for 7 days after initiation of arthritis had a similar effect. CII 1, 10, 100 µg/ml had no effect on IL-1 and TNF production by synoviocytes *in vitro*, but CII 10 µg/ml suppressed IL-1 and TNF production by synoviocytes-PP cells co-culture system ( $P<0.01$ ), which was antagonized by anti-TGF- $\beta$  antibody (10 µg/ml) ( $P<0.01$ ). Portal serum (1:10) from oral tolerized rats suppressed IL-1 and TNF production by synoviocytes ( $P<0.01$ ), which was also antagonized by anti-TGF- $\beta$  antibody (10 µg/ml) ( $P<0.01$ ).

**Conclusions:** Oral administration of CII had prophylactic and therapeutic effects on AA and over-production of IL-1, TNF, NO and MDA by synoviocytes was

suppressed. Bystander active suppression may be the main mechanism of oral CII in the suppression of synoviocyte function.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by hyperplasia of synovium with resultant cartilage destruction. Proliferation of the synovium is associated with increased production of pro-inflammatory cytokines, mainly interleukin (IL)-1 and tumour necrosis factor (TNF), which play crucial roles in cartilage degradation [1,2]. Nitric oxide (NO) and oxygen radicals also play an important role in chondrocyte catabolic activity [3]. Therefore, suppression of production of these pro-inflammatory mediators by synovial cells may be an important target of therapy for RA.

Oral tolerance, a state of immunological unresponsiveness induced by oral administration of antigen, has posed intriguing possibilities for the treatment of autoimmune diseases including RA, multiple sclerosis and insulin dependent diabetes [4-8]. Oral tolerance has been applied to prevent and treat autoimmune disease in several animal models including arthritis. Oral administration of type II collagen (CII) has been shown to suppress collagen [9-11], adjuvant [12], antigen [13] and pristane [14] induced arthritis in mice and rats. However, the precise mechanisms of oral tolerance are not fully known. Studies of experimental autoimmune encephalomyelitis (EAE) have shown that oral tolerance of myelin basic protein (MBP) was associated with down-regulation of pro-inflammatory cytokines such as IL-1 and TNF expressions and up-regulation of suppressive cytokines such as transforming growth factor (TGF)- $\beta$  and IL-4 expressions in brain tissue [15]. It is generally agreed that the regulation of mucosally induced tolerance involves several mechanisms, including active suppression induced by low doses of antigen and clonal anergy or deletion induced by high doses of antigen [4-8,16]. Cells from Peyer's patches (PP) in the gut-associated lymphoid tissue (GALT) and TGF- $\beta$  are reported to mediate the induction

of active suppression [17,18]. But there are few reports about the mechanisms of mucosal tolerance to CII in animal models of arthritis [11 -13, 19-22], especially its effect on synoviocytes.

Adjuvant arthritis (AA) is a commonly used model of human RA with an incidence of around 90%, which makes it ideal to investigate anti-inflammatory effects. In the present study, therefore, we tested the effect of oral administration of CII on pro-inflammatory mediator production by synoviocytes in rats with AA.

## MATERIALS AND METHODS

**Animals** Male Sprague-Dawley (SD) rats weighing 150-190 g were obtained from Animal Centre, Anhui Medical University (China). They were housed five per cage and fed a standard laboratory chow and water *ad libitum*. Inbred C<sub>57</sub>BL/6J mice weighing 16-20 g were also obtained from this Animal Centre.

**Induction and evaluation of AA** Complete Freund's adjuvant (CFA) was prepared by suspending heat-killed *mycobacterium tuberculosis* (MT) in liquid paraffin at 10 mg/ml. AA was induced by a single intradermal injection of 100 µl of CFA into the left hind paw. At day 0, 16, 20, 24 and 28 after immunization, the right hind paw volume was measured with a water replacement plethysmometer (Mukomachi K iai CD, Japan). Paw swelling ( $\Delta$ ml) was calculated by taking away the paw volume at day 0 from the related one at day 16, 20, 24 and 28.

**Oral administration of CII** In the pre-treatment protocol, rats were fed daily doses of 5, 50 or 500 µg/Kg of bovine CII (provided by Qilu Pharmaceutical Company, China) dissolved in 0.01 M acetic acid using an 18 -gauge stainless steel animal -feeding needle for 7 days prior to immunization. Control rats were only fed the same volume of vehicle (0.01 M acetic acid). In the treatment protocol, rats were treated orally with above doses of CII or vehicle for 7 days from day 13 after immunization, for paw swelling appeared on day 10-13.

**Synovial cell isolation and cultures** Synovial cells (synoviocytes) were isolated as described [23,24]. After measurement of paw swelling, rats were killed at day 30 after immunization, and the synovial tissue from right ankle was excised. The synovial membranes were minced aseptically, then dissociated enzymatically using collagenase (4.0 mg/ml, Sigma) in Dulbecco's modified Eagle's medium (DMEM, GIBCO) for 4 h at 37 °C. After centrifugation for 10 minutes at 500 g, cells were

resuspended in DMEM with 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml gentamicin, 20 mM HEPES buffer, and 10% fetal calf serum (FCS). Cells were cultured in 50 ml culture dishes in humidified 5% CO<sub>2</sub> atmosphere at 37 °C. After adherence for 18 h, cells were washed thoroughly with phosphate-buffered saline (PBS) solution. Adherent synovial cells were removed by adding trypsin-EDTA followed by washing with PBS containing 2% FCS. Synovial cells collected from different groups were used in 4-5 passages for subsequent experiments, when they showed fibroblastoid morphology. They ( $5 \times 10^5$ /well) were cultured for 48 h with 5 µg/ml lipopolysaccharide (LPS, Sigma) and the supernatants were collected for measurement.

**Serum collection from oral tolerized rats** Rats were treated orally with CII 5, 50 or 500 µg/Kg or vehicle daily for 7 days. Two days later, blood samples were obtained from portal vein while rats were under light ether anesthesia. After centrifugation, pooled serum was added to culture medium of synovial cells, and the dilution of 1:10 was found to be most effective on synoviocyte function.

***In vitro* experiments** Synovial cells obtained from AA rats were used in 4-5 passages for subsequent *in vitro* experiments. They ( $5 \times 10^5$ /well) were cultured for 48 hours in 24-well plates containing DMEM with 10% FCS and 5 µg/ml LPS in absence or presence of CII (1, 10, 100 µg/ml), 1:10 serum (from non- or tolerized rats) or anti-TGF-β antibody (R & D Systems, 10 µg/ml) in humidified 5% CO<sub>2</sub> atmosphere at 37 °C. Supernatants were collected for measurement.

Peyer's patches (PP) were harvested from normal rats and pooled, minced into single cell suspensions through nylon mesh in DMEM, and washed 3 times. Then PP cells ( $10^6$ /well) were co-cultured with synovial cells ( $5 \times 10^5$ /well) for 48 hours in 24-well plates containing DMEM with 10% FCS and 5 µg/ml LPS in absence or presence of

CII (10 µg/ml) or anti-TGF-β antibody (10 µg/ml) in humidified 5% CO<sub>2</sub> atmosphere at 37 °C. Supernatants were collected for measurement.

**IL-1 activity bioassay** IL-1 activity was measured by a mouse thymocyte activation assayed by MTT dye reduction [25,26]. The tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) was dissolved in sterile PBS to a concentration of 5 mg/ml and stored in the dark at 4 °C for up to 1 week. Immediately before use, stock MTT was filtered (0.22 µm) to remove any formazan precipitate. Thymocytes (2×10<sup>6</sup>/well) from mice were cultured for 48 hours in 96-well plates containing RPMI 1640 medium (GIBCO) supplemented with 5 µg/ml concanavalin A (ConA, Sigma) and 0.1 ml collected supernatants in triplicate. Three hours before the termination of culture, cells were pulsed with MTT stock (20 µl/well), returned to 37 °C and incubated for an additional 3h. The plates were centrifuged for 10 min at 1,000 g to pellet cells and MTT formazan product. The supernatant was carefully aspirated without disturbing the pellet, and formazan was solubilized by addition of isopropanol (100 µl/200µl supernatant). Insoluble material was then removed by centrifugation for 10 min at 1,000 g. The solubilized formazan in isopropanol was collected and distributed into 12-well, flat-bottom ELISA plates at a final volume of 100 µl/well. Plates were read at 570 nm in EL 301 Strip Reader (Bio-Tek, USA) within 1 h of addition of isopropanol. Values were expressed as mean absorbance (A) of triplicate wells.

**TNF bioassay** TNF activity in the supernatants was measured by an L929 cytotoxicity bioassay [27,28]. Briefly, L929 cells (obtained from Department of Immunology, Beijing University) were cultured in RPMI 1640 medium with 5% FCS at 10<sup>5</sup> cells/well for 24 h in 96-well, flat-bottom plates at 37 °C in a 5% CO<sub>2</sub> atmosphere. The medium above the non-confluent cell layer was replaced with fresh



medium containing actinomycin D (4 µg/ml, Sigma) and serial dilutions of supernatants to be tested for TNF activity. All samples were tested in triplicate. After a further 24 hour incubation the medium was removed and a 0.5% in 20% methanol solution of crystal violet was added to the wells. The absorbance in each well was assessed with a plate reader at 570 nm. Cell lysis was calculated as follows: Percent lysis=(A-B)/A × 100. A is the absorbance of L929 cells cultured only with complete medium and B is the absorbance of L929 cells cultured with a test samples.

**Nitrite estimation** Nitrite concentration represented NO level in the supernatant was measured as described before [29]. Briefly, 100 µl aliquots were removed from collected supernatants and incubated with an equal volume of Griess reagent (1% sulphanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H<sub>3</sub>PO<sub>4</sub>) at room temperature for 10 min. The absorbance at 550 nm was determined in a plate reader. A standard curve was established with a set of serial dilutions of sodium nitrite. All samples were assayed in triplicate. Results were expressed as µmol/l.

**MDA estimation** Malondialdehyde (MDA) levels represented lipid peroxidation in supernatants, were determined using the method described before [30]. Briefly, MDA was reacted with thibarbituric acid (TBA) by incubating for 1 h at 95 -100 °C. Following the reaction, absorbance was measured with a spectrophotometry at 535 nm. Results were expressed as µmol/l.

**Statistical analysis** Results were expressed as mean ± SD. Student's t-test was used to make comparisons between the groups. P values <0.05 were considered statistically significant.

## **RESULTS**

### **CII oral pre-treatment suppresses AA and synoviocyte function**

To determine if oral administration of CII has prophylactic effect on AA, AA rats were pre-fed with vehicle or three doses of CII for 7 days before immunization. As shown in Table 1, AA rats pre-fed with vehicle had significant hind paw secondary swelling measured at day 16, 20, 24 and 28 after immunization with CFA. Daily pre-feeding with CII 5, 50 and 500 µg/Kg significantly suppressed AA at day 16, 20, 24 and 28 after immunization (all  $P < 0.01$ ) with the most pronounced effects seen in the group fed with 50 µg/Kg of CII. The suppression rates ranged between 33.9% and 58.4%.

To investigate the effects of pre-feeding with CII on synoviocyte function in AA rats, the rats were decapitated at day 30 after immunization and IL-1, TNF, NO and MDA levels produced from synoviocytes were measured. As shown in Table 2, IL-1, TNF, NO and MDA produced from synoviocytes increased significantly in AA rats as compared with those in normal rats (all  $P < 0.01$ ). Daily pre-feeding of 3 doses of CII had significant inhibitory effects on IL-1, TNF, NO and MDA production from synoviocytes as compared with daily pre-feeding with vehicle (all  $P < 0.01$ ) in AA rats. The most pronounced effect was seen in the group fed with 50 µg/Kg of CII.

### **CII oral treatment suppresses AA and synoviocyte function**

To test if oral administration of CII has therapeutical effect on AA, AA rats were fed with vehicle or three doses of CII for 7 days from day 13 after immunization. As shown in Table 3, daily oral treatment with CII 5, 50 and 500 µg/Kg also significantly suppressed hind paw secondary swelling measured at day 20, 24 and 28 after immunization (all  $P < 0.01$ ). The suppression rates ranged between 20.2% and 35.8%.

To examine the effects of oral treatment with CII on synoviocyte function in AA rats, the rats were decapitated at day 30 after immunization and IL-1, TNF, NO and MDA produced from synoviocytes were measured. As shown in Table 4, daily feeding of 3 doses of CII had significant inhibitory effects on IL-1, TNF, NO and MDA production from synoviocytes as compared with daily feeding with vehicle (all  $P<0.01$ ) in AA rats. The most pronounced effect was also seen in the group fed with 50  $\mu\text{g/Kg}$  of CII.

### **CII suppresses synoviocyte function via PP cells and TGF- $\beta$ *in vitro***

To test if CII has a direct effect on synoviocytes, *in vitro* experiments were employed. As shown in Figure 1, CII 1, 10, 100  $\mu\text{g/ml}$  had no effects on IL-1 and TNF production by synoviocytes of AA rats *in vitro*.

As CII has been shown to stimulate lymphocyte proliferation *in vitro* [31, Ding CH et al. unpublished data], we investigated if CII acts on synoviocytes via lymphocytes and TGF- $\beta$  secreted by Th3 cells. As shown in Figure 2, CII 10  $\mu\text{g/ml}$  had no effect on IL-1 and TNF production from synoviocytes of AA rats *in vitro* (group 2 vs group 1,  $P>0.05$ ). PP cells ( $10^6/\text{well}$ ) from normal rats also had no effect on IL-1 and TNF production when co-cultured with synoviocytes ( $5\times 10^5/\text{well}$ ) from AA rats (group 3 vs group 1,  $P>0.05$ ). However, CII 10  $\mu\text{g/ml}$  suppressed IL-1 and TNF production significantly in the co-culture system (group 4 vs group 2 and 3,  $P<0.01$ ). Meanwhile, anti-TGF- $\beta$  antibody (10  $\mu\text{g/ml}$ ) was added and it was demonstrated that anti-TGF- $\beta$ , which had no effect on IL-1 and TNF production by synoviocytes (group 5 vs group 2,  $P>0.05$ ), significantly antagonised the suppressive effects of CII on IL-1 and TNF production in the co-culture system (group 6 vs group 4,  $P<0.01$ ).

### **Serum from oral tolerized rats suppresses synoviocyte function *in vitro***

To determine if the suppressive effects of oral administration of CII on synoviocyte function are through blood mediators, we collected the portal vein sera from the rats fed with CII 5, 50 or 500  $\mu\text{g/Kg}$  or vehicle (as a control) daily for 7 days and tested the effects of these sera on synoviocyte function *in vitro*. As shown in Figure 3, as compared with serum (1:10) from rats fed with vehicle, serum (1:10) from rats fed with CII 5, 50 or 500  $\mu\text{g/Kg}$  significantly inhibited IL-1 and TNF production from synoviocytes of AA rats (all  $P < 0.01$ ).

Furthermore, to test if the inhibitory effects of these sera are via TGF- $\beta$  in blood, we added anti-TGF- $\beta$  antibody (10  $\mu\text{g/ml}$ ) together with serum from rats fed with CII 50  $\mu\text{g/Kg}$  or vehicle into the synoviocytes culture medium. As shown in Figure 4, serum (1:10) from rats fed with CII 50  $\mu\text{g/Kg}$  had inhibitory effects on IL-1 and TNF production from synoviocytes of AA rats (group 2 vs group 1,  $P < 0.01$ ), which antagonized by anti-TGF- $\beta$  (group 4 vs group 2,  $P < 0.01$ ).

## DISCUSSION

Our study demonstrated that oral CII pre-feeding or feeding suppressed secondary hind paw swelling and pre-inflammatory mediators production in AA. CII had no direct effect on synoviocyte function *in vitro*, but it could suppress IL-1 and TNF production by synoviocytes via TGF- $\beta$  secreted by PP cells. Furthermore, portal serum from oral tolerized rats suppressed IL-1 and TNF production by synoviocytes via TGF- $\beta$  *in vitro*.

Beginning with collagen-induced arthritis (CIA) in rats [10] and mice [9], oral administration of CII has been found to suppress virtually all experimentally inducible animal models that exist for RA [11-14, 32]. Histopathological studies showed that oral administration of CII resulted in reduction of synovial hyperplasia, mononuclear infiltration, pannus formation, and cartilage erosions [14]. In AA models, using Lewis rats weighing between 125 to 150 grams, daily doses of 3 or 30  $\mu$ g of native chicken CII abrogated the disease to a statistically significant degree, whereas higher doses or a dose of 0.3  $\mu$ g/day were ineffective. Both pre-feeding and feeding suppressed the disease of AA [12]. In our present study, using 150-190 g SD rats, we found that daily oral administration of bovine CII 5, 50 and 500  $\mu$ g/kg for 7 days either before or after immunization suppressed secondary hind paw swelling of AA. Our results and doses are consistent with the previous findings [12]. Moreover, we demonstrated, for the first time, that oral administration of CII either before or after immunization inhibited the production of pro-inflammatory mediators, i.e., IL-1, TNF, NO and MDA, from synoviocytes in AA. These results suggest that suppression of paw swelling by oral CII may be through suppression of pro-inflammatory mediator production by synoviocytes. This implies that oral administration of CII could modify the disease

activity of arthritis, for these pro-inflammatory mediators play crucial roles in cartilage degradation in inflammatory arthritis [1 -3]. Although the effects of oral administration of CII in the treatment of RA from clinical trials are inconsistent, showing no effect, non significant, or significant improvement in the disease[8, 33-36], our present study in AA supports the concept that OT induction by CII has a potential prospect in modifying disease activity of RA.

So far there has not been a convincing explanation why oral administration of CII suppresses synovial inflammation. In other animal systems a number of studies have pinpointed the ways by which OT is likely to be achieved [4 -8, 37-39]. It has been observed in mice and rats that multiple feedings of low doses of a soluble antigen leads to development of active suppression and regulatory T cells (CD4<sup>+</sup> Th2 and Th3 cells) in GALT containing PP. Th2 cells secrete IL -4 and IL-10, whereas Th3 cells secrete TGF-  $\beta$  . These regulatory T cells migrate to lymphoid organs and target organs expressing the same antigen . Antigen exposure stimulates cells from these organs to secrete anti-inflammatory cytokines (TGF-  $\beta$  , IL-4, and IL-10). Multiple feedings of low doses of a soluble antigen also induced splenic CD4<sup>+</sup> CD25<sup>+</sup> regulatory cells to produce the anti-inflammatory cytokines [40,41]. These cytokines downregulate other T-cells responses in the organ in which the autoimmune attack occurs. The ability to suppress immune response in the microenvironment in an antigen non-specific manner is known as bystander suppression [ 4-8, 37-39]. In the course of bystander suppression induction, PP cells and TGF -  $\beta$  secreted by Th3 or CD4<sup>+</sup>CD25<sup>+</sup> cells play important roles. PP cells have been used to transfer tolerance to soluble antigen [42]. CD4<sup>+</sup> and CD8<sup>+</sup> regulatory cells were detected in PP as early as 6 h following oral antigen in TCR transgenic mice [43]. PP -null mice lack

ovalbumin-specific OT suggests PP cells are required in OT [17]. Similarly, TGF- $\beta$  is an important mediator of the active component of OT. Increased production of this cytokine has been reported in a number of models of OT and TGF- $\beta$ -secreting Th3 or CD4<sup>+</sup>CD25<sup>+</sup> T cells can be produced from animals tolerized in this way [4 -8, 40]. Administration of anti-TGF- $\beta$  antibody in vivo negates the ability of oral MBP to protect against induction of EAE [18].

The roles of PP cells and TGF- $\beta$  in OT were verified by our present *in vitro* study. We found that CII (1, 10 and 100  $\mu$ g/ml) had no direct effect on IL-1 and TNF production by synoviocytes. However, when PP cells were added to the culture system, CII 10  $\mu$ g/ml significantly inhibited IL-1 and TNF production by synoviocytes. This effect of CII was antagonized by anti-TGF- $\beta$  antibody (10  $\mu$ g/ml). Our findings strongly suggest that bystander active suppression is the main mechanism of OT to CII, which is consistent with previous reports [11-13,19-22]. It has been reported that spleen cells, and nylon wool-filterer splenic T cells from CII-induced OT rats adoptively transferred hyporesponsiveness to normal recipients that were less susceptible to AA or CIA [12, 19], suggesting regulatory T cells mediate the bystander active suppression. T cells lines established from CII fed or nasally treated mice showed increased production of IL-2, IL-10 and TGF- $\beta$ , and decreased production of IFN- $\gamma$ . Meanwhile, TNF- $\alpha$  and IL-6 mRNA expressions in the joint were diminished by nasal treatment of CII in mice with CIA [11], showing CII induced mucosal tolerance could suppress local inflammation of joint in CIA. Neutralization of IL-4 by an anti-IL-4 antibody prevented the suppression of CIA in mice by oral administration of CII, implying that IL-4 may play a role in its

suppression [22]. TGF- $\beta$  1 increased the degree of OT induced by feeding of low - dose CII in mice and augmented the induction of immunoregulatory CD8 T cells, which transferred the resistance to CIA induction to normal recipients [18]. Single administration of PLGA (a carrier for drug delivery systems) entrapping CII showed a higher level of TGF- $\beta$  mRNA expression in PP, but a lower level of TNF- $\alpha$  mRNA expression in draining lymph nodes in CIA mice [44], suggesting TGF- $\beta$  plays an important role in OT induction by CII.

Surgical approaches using portacaval shunts showed that the development of OT is dependent on the hepatic portal circulation [45,46], so we studied the effect of the serum in portal vein from oral tolerized rats. We found that the serum suppressed IL -1 and TNF production by synoviocytes *in vitro*, which was antagonized by anti-TGF- $\beta$  antibody. This result suggests that TGF- $\beta$  level in the hepatic portal circulation may also play a role in suppression of synovial inflammation by oral administration of CII.

In conclusion, oral administration of CII had prophylactic and therapeutic effects on AA and over-production of IL-1, TNF, NO and MDA by synoviocytes was suppressed. CII had no direct effect on synoviocytes, but it could suppress IL -1 and TNF production by synoviocytes via TGF- $\beta$  secreted by PP cells *in vitro*. Portal vein serum from oral tolerized rats also suppressed IL-1 and TNF production by synoviocytes via TGF- $\beta$  *in vitro*. Our study suggests that bystander active suppression may be the main mechanism of OT to CII in the suppression of synovial inflammation.



**Acknowledgement** This work was awarded Servier Young Investigator Prize in Pharmacology by the Chinese Pharmacological Society and the Institute de Recherches Internationales Servier (France). A special thanks to Ms Lili Zhang who provided CII to us.

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**Table 1 Inhibitory effects of oral CII pre-treatment on hind paw swelling in AA rats**

Group	Dose μg /kg	Paw Swelling (Δml)			
		day16	day 20	day 24	day 28
Normal	-	0.10±0.02	0.15±0.02	0.20±0.02	0.23±0.02
AA	-	0.46±0.09**	0.67±0.12**	0.87±0.14**	1.05±0.12**
CII	5	0.30±0.07†† (33.9)	0.37±0.07†† (49.9)	0.42±0.08†† (51.2)	0.48±0.08†† (54.1)
	50	0.20±0.02†† (56.1)	0.30±0.04†† (55.9)	0.36±0.04†† (58.4)	0.45±0.03†† (57.5)
	500	0.27±0.04†† (44.3)	0.38±0.03†† (44.0)	0.45±0.02†† (47.6)	0.54±0.02†† (49.0)

SD rats were fed with vehicle (0.01 M acetic acid) or different daily doses of bovine CII for 7 days prior to immunization. Adjuvant arthritis (AA) was induced by a single intradermal injection of 100 μl of Complete Freund's Adjuvant into the left hind paw. The right hind paw volume was measured with a water replacement plethsmometer at day 0, 16, 20, 24 and 28 after immunization. Paw swelling (Δml) was calculated by taking away the paw volume at day 0 from the relevant one at day 16, 20, 24 and 28. Results are representative of two similar experiments and expressed as mean ± SD, n=5. \*\*P<0.01 vs Normal group, ††P<0.01 vs AA group. Numbers in parenthesis represent the inhibitory percentages.

**Table 2. Effects of oral CII pre-treatment on synoviocyte function in AA rats**

Group	Dose	IL-1	TNF	NO	MDA
	μg /kg	(A)	(%)	(μmol/l)	(μmol/l)
Normal	-	0.44±0.05	28.92±9.81	5.84±1.29	0.63±0.18
AA	-	0.67±0.07**	65.54±6.49**	12.14±1.09**	1.86±0.27**
CII	5	0.48±0.05††	38.46±9.36††	6.89±1.07††	0.96±0.34††
	50	0.49±0.06††	32.61±8.86††	6.46±0.64††	0.61±0.17††
	500	0.53±0.05††	39.08±10.12††	6.91±1.39††	0.72±0.22††

SD rats were fed with vehicle (0.01 M acetic acid) or different daily doses of bovine CII for 7 days prior to immunization. Adjuvant arthritis (AA) was induced by a single intradermal injection of 100 μl of Complete Freund's Adjuvant into the left hind paw. At day 30 after immunization, rats were killed and synoviocytes were isolated and cultured. IL-1, TNF, NO and MDA secreted from synoviocytes were measured as described in *materials and methods*. Results are representative of two similar experiments and expressed as mean ± SD, n=5. \*\*P<0.01 vs Normal group, ††P<0.01 vs AA group.



**Table 3. Inhibitory effects of oral CII treatment on hind paw swelling in AA rats**

Group	Dose $\mu\text{g /kg}$	Paw Swelling ( $\Delta\text{ml}$ )		
		day 20	day 24	day 28
Normal	-	0.15 $\pm$ 0.04	0.23 $\pm$ 0.06	0.32 $\pm$ 0.07
AA	-	0.53 $\pm$ 0.07**	0.77 $\pm$ 0.03**	0.94 $\pm$ 0.07**
CII	5	0.43 $\pm$ 0.09 $\dagger\dagger$ (20.2)	0.56 $\pm$ 0.04 $\dagger\dagger$ (27.6)	0.65 $\pm$ 0.06 $\dagger\dagger$ (30.5)
	50	0.37 $\pm$ 0.06 $\dagger\dagger$ (30.9)	0.57 $\pm$ 0.06 $\dagger\dagger$ (20.6)	0.41 $\pm$ 0.08 $\dagger\dagger$ (34.5)
	500	0.37 $\pm$ 0.05 $\dagger\dagger$ (31.1)	0.49 $\pm$ 0.09 $\dagger\dagger$ (35.8)	0.62 $\pm$ 0.06 $\dagger\dagger$ (34.2)

Adjuvant arthritis (AA) was induced by a single intradermal injection of 100  $\mu\text{l}$  of Complete Freund's Adjuvant into the left hind paw. Vehicle (0.01 M acetic acid) or different daily doses of bovine CII were fed for 7 days from d 13 after immunization. The right hind paw volume was measured with a water replacement plethsmometer at day 0, 20, 24 and 28 after immunization. Paw swelling ( $\Delta\text{ml}$ ) was calculated by taking away the paw volume at day 0 from the relevant one at day 20, 24 and 28. Results are representative of two similar experiments and expressed as mean  $\pm$  SD, n=5. \*\*P<0.01 vs Normal group,  $\dagger\dagger$ P<0.01 vs AA group. Numbers in parenthesis represent the inhibitory percentages.

**Table 4 Inhibitory effects of oral CII treatment on synoviocyte functions in AA rats**

Group	Dose	IL-1	TNF	NO	MDA
	$\mu\text{g /kg}$	(A)	(%)	( $\mu\text{mol/l}$ )	( $\mu\text{mol/l}$ )
Normal	-	0.46 $\pm$ 0.06	28.31 $\pm$ 9.40	7.25 $\pm$ 1.38	0.80 $\pm$ 0.24
AA	-	0.69 $\pm$ 0.04**	64.00 $\pm$ 11.39**	13.61 $\pm$ 1.56**	2.19 $\pm$ 0.32**
CII	5	0.50 $\pm$ 0.06††	47.07 $\pm$ 5.92†	8.81 $\pm$ 1.24††	1.02 $\pm$ 0.28††
	50	0.47 $\pm$ 0.05††	40.31 $\pm$ 9.81††	6.74 $\pm$ 0.73††	0.81 $\pm$ 0.17††
	500	0.51 $\pm$ 0.04††	49.85 $\pm$ 5.17†	7.48 $\pm$ 0.68††	0.93 $\pm$ 0.25††

Adjuvant arthritis (AA) was induced by a single intradermal injection of 100  $\mu\text{l}$  of Complete Freund's Adjuvant into the left hind paw. Vehicle (0.01 M acetic acid) or different daily doses of bovine CII were fed for 7 days from day 13 after to immunization. At day 30 after immunization, rats were killed and synoviocytes were isolated and cultured. IL-1, TNF, NO and MDA secreted from synoviocytes were measured as described in *materials and methods*. Results are representative of two similar experiments and expressed as mean  $\pm$  SD, n=5. \*\*P<0.01 vs Normal group, ††P<0.01 vs AA group.

**Figure legends:**

**Figure 1. Effects of CII on IL-1 and TNF production by synoviocytes of AA rats**

*in vitro*. Synovial cells ( $5 \times 10^5$ /well) obtained from AA rats on day 30 after immunization were cultured for 48 hours in 24-well plates containing DMEM with 10% FCS and 5 µg/ml LPS in absence or presence of CII (1, 10, 100 µg/ml). Supernatants were collected for IL-1 and TNF measurement. Results are expressed as mean  $\pm$  SD of 5 independent experiments.

**Figure 2. Effects of CII on IL-1 and TNF production by synoviocytes co-cultured**

**with PP cells *in vitro* and antagonism of anti-TGF- $\beta$  antibody.** Peyer's patches (PP) were harvested from normal rats and synovial cells were obtained from AA rats on day 30 after immunization. PP cells ( $10^6$ /well) were co-cultured with synovial cells ( $5 \times 10^5$ /well) for 48 hours in 24-well plates containing DMEM with 10% FCS and 5 µg/ml LPS in absence or presence of CII (10 µg/ml) or anti-TGF- $\beta$  antibody (10 µg/ml). Supernatants were collected for IL-1 and TNF measurement. '+' represents 'added', '-' represents 'not added'. Results are expressed as mean  $\pm$  SD of 5 independent experiments. \*\*P<0.01 vs group 2, ##P<0.01 vs group 4.

**Figure 3. Effects of serum from oral tolerized rats on IL-1 and TNF production**

**by synoviocytes of AA rats *in vitro*.** Sera in portal vein were obtained from rats treated orally with CII 5, 50 or 500 µg/Kg daily or vehicle for 7 days. Synovial cells ( $5 \times 10^5$ /well) obtained from AA rats were cultured for 48 hours in 24-well plates containing DMEM with 10% FCS and 5 µg/ml LPS in absence or presence of the different serum (1:10). Supernatants were collected for IL-1 and TNF measurement. Results are expressed as mean  $\pm$  SD of 5 independent experiments. \*\*P<0.01 vs cells treated with serum from vehicle-fed rats.

**Figure 4. Antagonism of anti-TGF-  $\beta$  antibody against the effects of serum from oral tolerized rats on IL-1 and TNF production by synoviocytes of AA rats *in vitro*.** Sera in portal vein were obtained from rats treated orally with CII 50  $\mu$ g/Kg daily or vehicle for 7 days. Synovial cells ( $5 \times 10^5$ /well) obtained from AA rats were cultured for 48 hours in 24-well plates containing DMEM with 10% FCS and 5  $\mu$ g/ml LPS in absence or presence of the different serum (1:10) or anti-TGF-  $\beta$  antibody (10  $\mu$ g/ml). Supernatants were collected for IL-1 and TNF measurement. '+' represents 'added', '-' represents 'not added'. Results are expressed as mean  $\pm$  SD of 5 independent experiments. \*\*P<0.01 vs group 1, ## P<0.01 vs group 2.

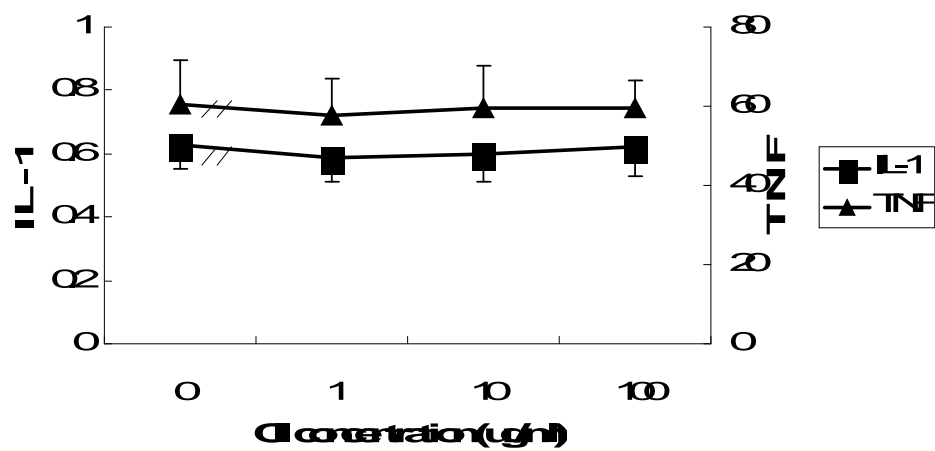
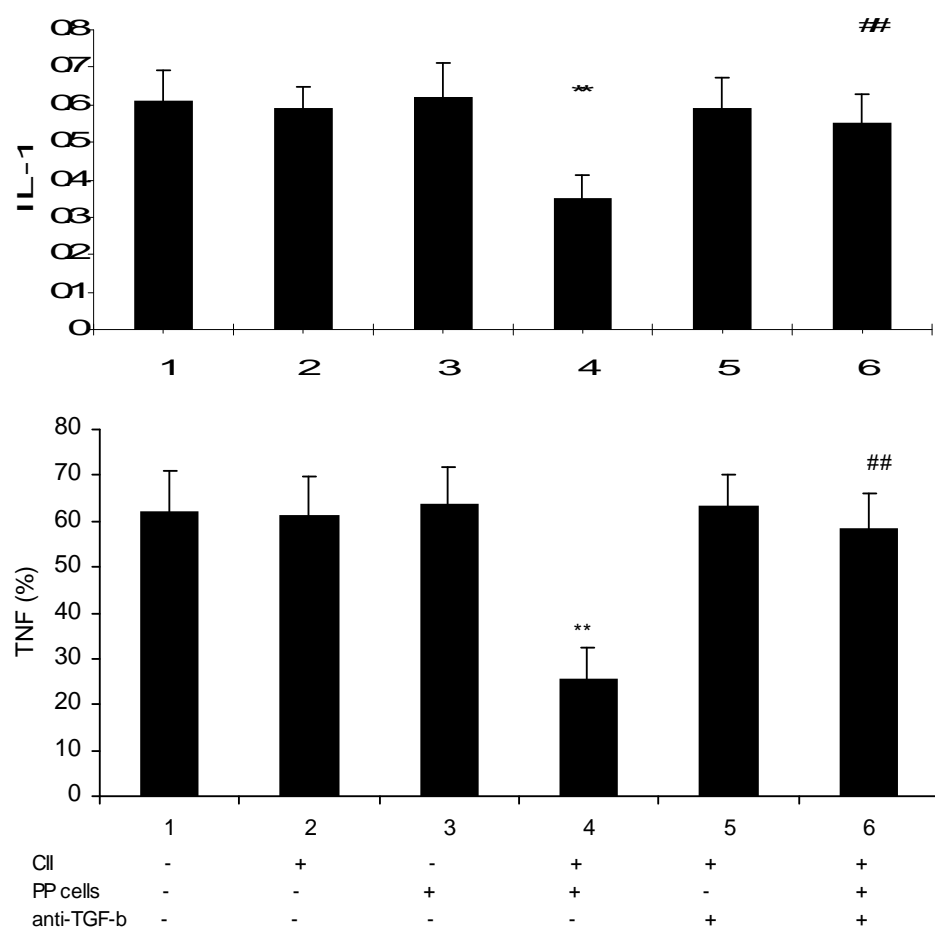


Figure 1.



**Figure 2**

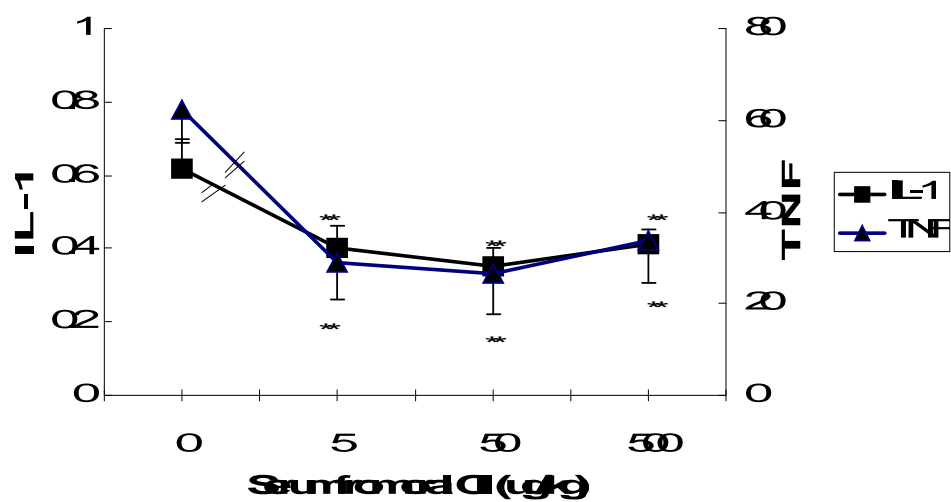
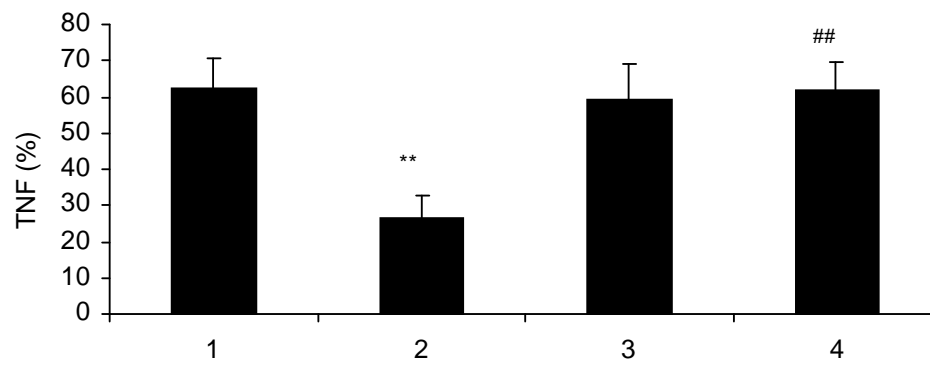
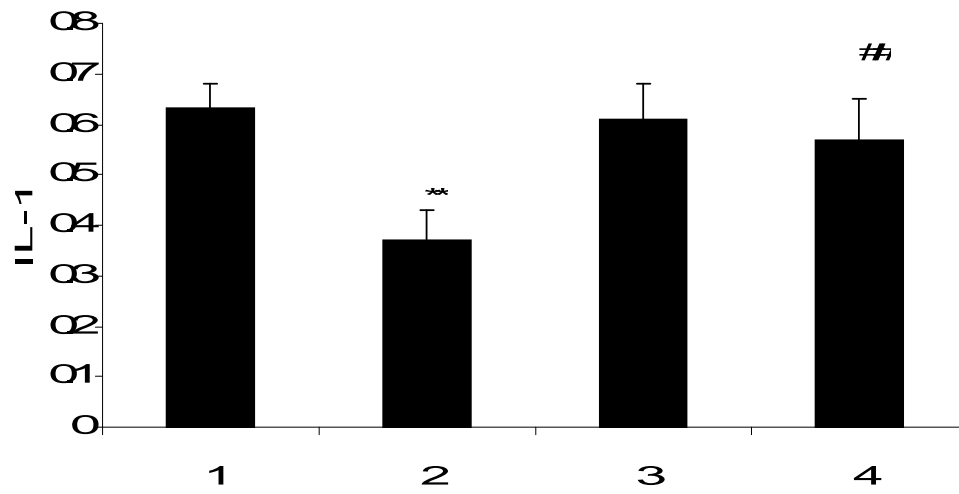


Figure 3



Serum from CII	-	+	-	+
Anti-TGF-b	-	-	+	+

**Figure 4**